# Improved Synthesis of 4-Cyanotryptophan and Other Tryptophan Analogues in Aqueous Solvent Using Variants of TrpB from *Thermotoga maritima*

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**Results of screening site-saturation mutagenesis libraries**

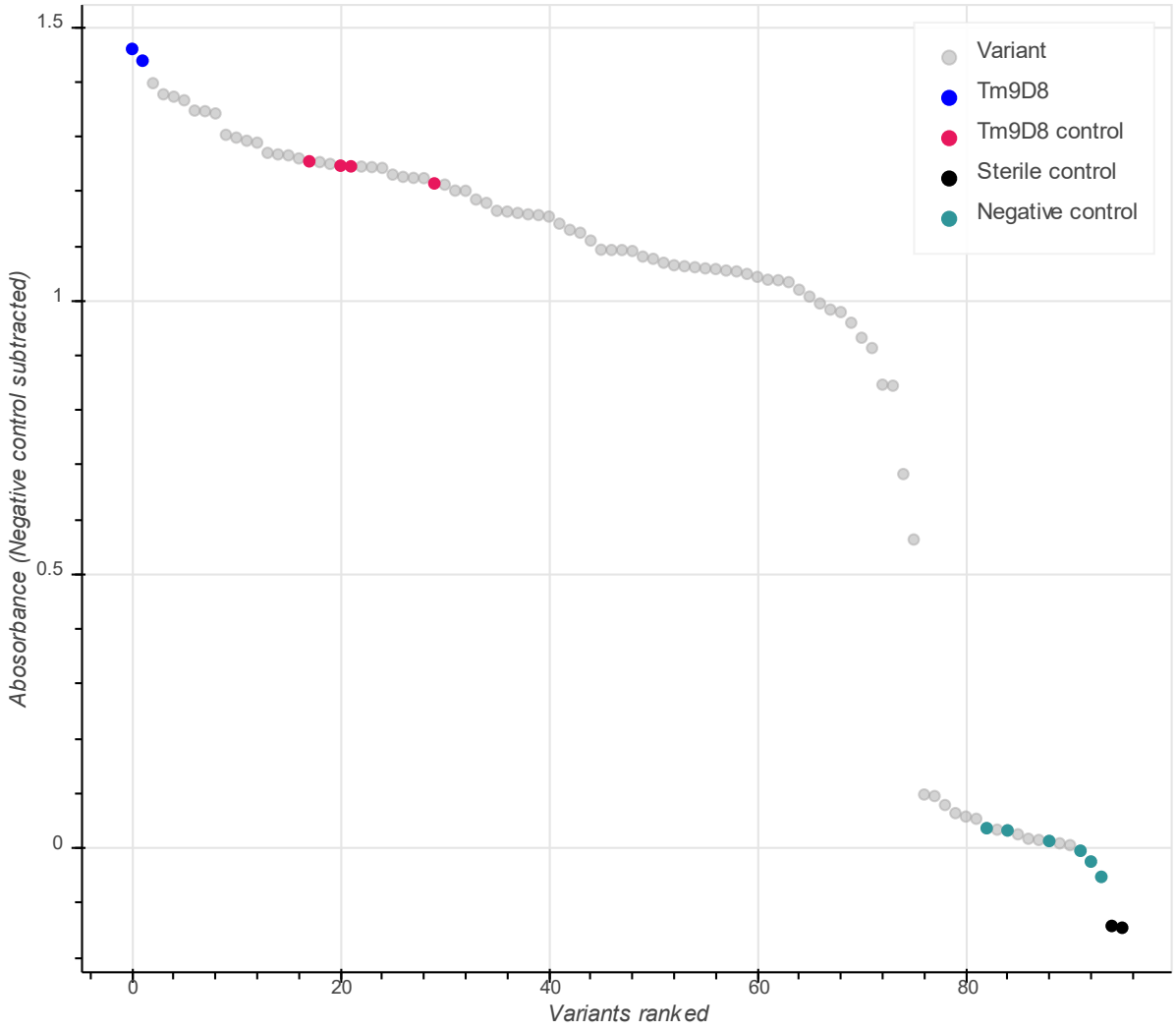



**Figure S1.** Site-saturation mutagenesis of residue 30 in Tm9D8. Following the procedure in the Experimental Section, 80 colonies (4-fold oversampling) were assayed for 4-CN-Trp production at 50 °C. Although many variants showed parent-like activity, the top two variants were found to be parent (G30).

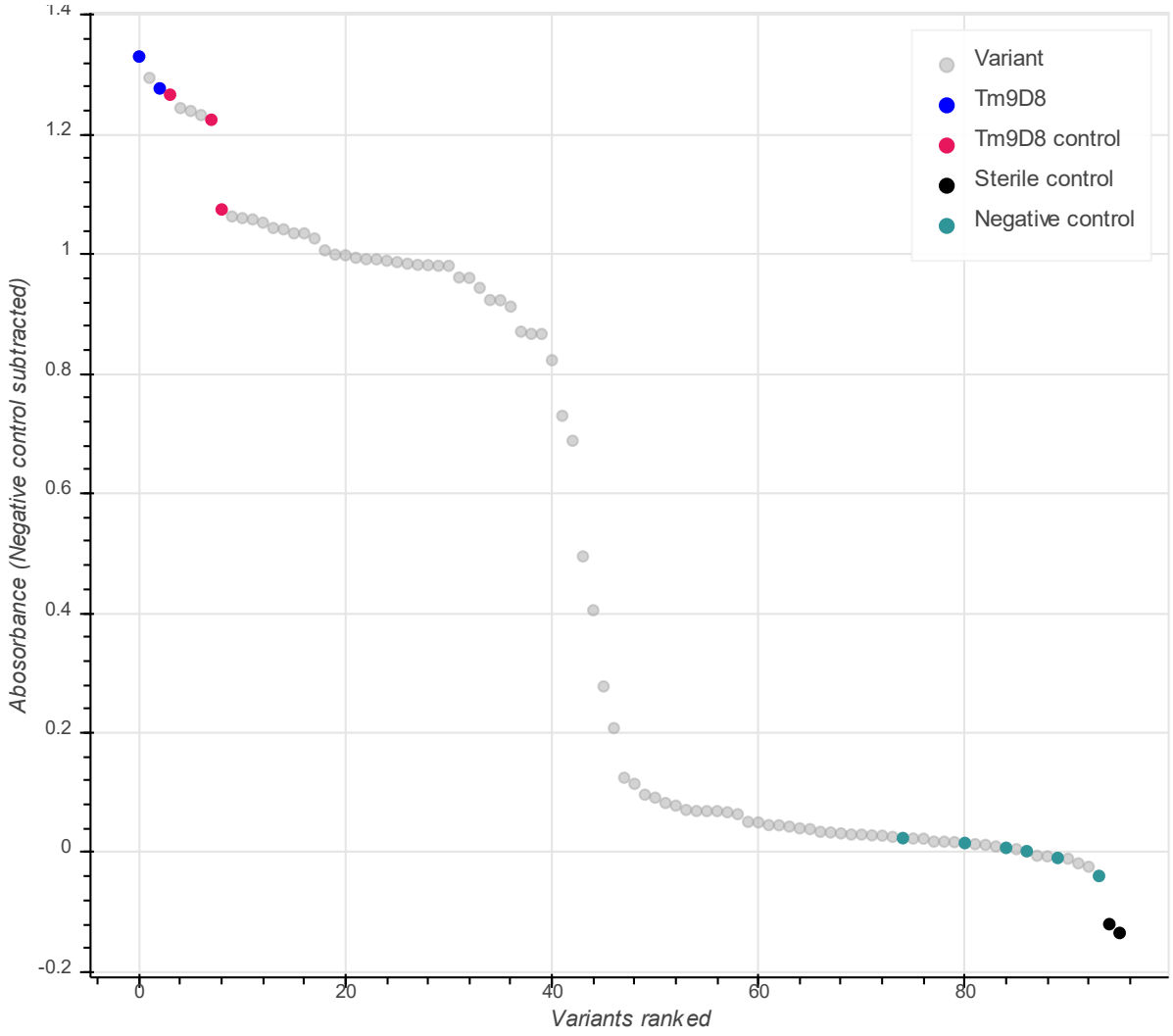



**Figure S2.** Site-saturation mutagenesis of residue 228 in *Tm*9D8. Following the procedure in the Experimental Section, 80 colonies (4-fold oversampling) were assayed for 4-CN-Trp production at 50 °C. The top two variants were identified as parent (S228).

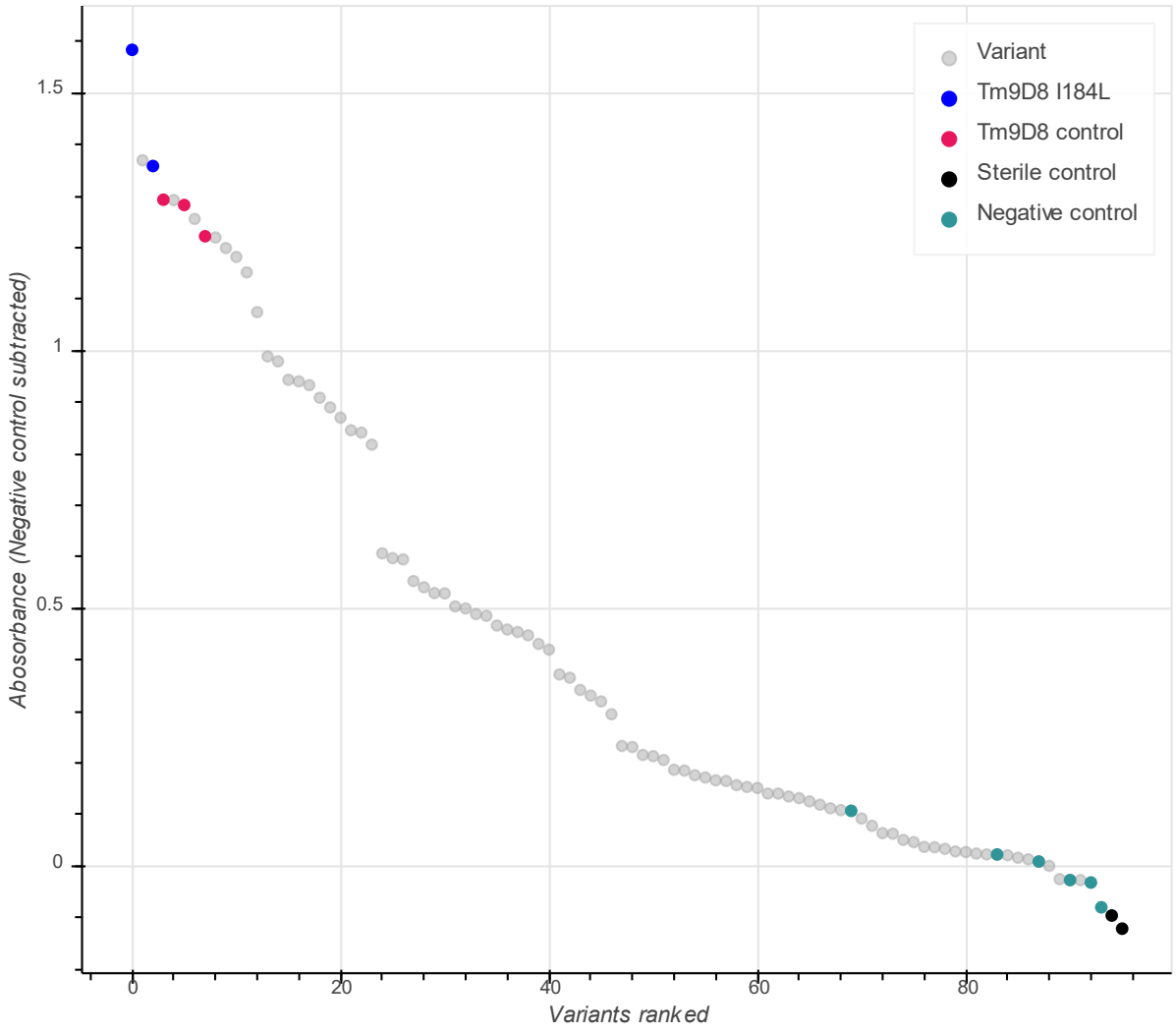



**Figure S3.** Site-saturation mutagenesis of residue 184 in Tm9D8. Following the procedure in the Experimental Section, 80 colonies (4-fold oversampling) were assayed for 4-CN-Trp production at 50 °C. I184L was found in two of the top variants, but this mutation was not as beneficial as I184F in the rescreen.

**LCMS calibration curves for Chart 1**

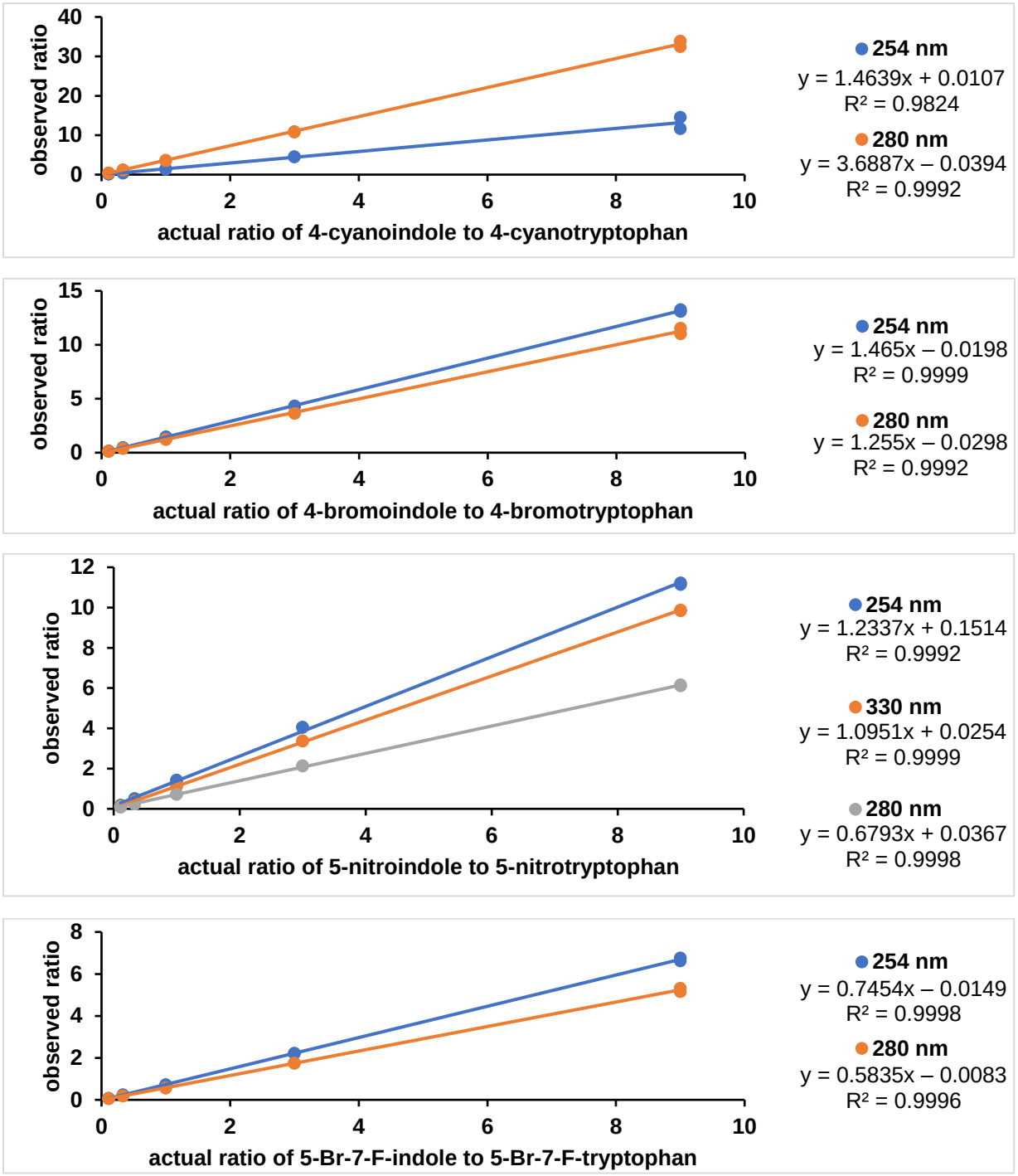



**Figure S4.** Using an authentic product standard, mixtures of starting material and product at different ratios (9:1, 3:1, 1:1, 1:3, and 1:9) were used to generate a calibration curve. Each mixture was prepared in duplicate with a final concentration of 1 mM in 1:1 1-M aq. HCl/ $CH_3CN$. Mixtures were analyzed with LCMS at 254 nm and 280 nm (reference 360 nm, bandwidth 100 nm) and 330 nm (no reference wavelength) and were correlated to the actual ratios by a linear relationship. The correlation for 4-nitrotryptophan was published previously.[1] For tryptophan and 5-chloro-7-iodotryptophan, the HPLC yield was approximated by comparing absorption peaks of product and starting material at 280 nm.

**Table S1. HPLC data for Figure 2**

| | Temperature (°C) | | | | | |
|---|---|---|---|---|---|---|
| | 37 | 37 | 50 | 50 | 75 | 75 |
| ***Tm*2F3** | | | | | | |
| 254 nm: | 5.68% | 5.76% | 17.1% | 17.3% | 15.1% | 18.7% |
| 280 nm: | 3.19% | 3.00% | 10.8% | 10.0% | 7.44% | 9.78% |
| ***Tm*9D8** | | | | | | |
| 254 nm: | 30.5% | 28.7% | 38.0% | 36.2% | 18.0% | 16.5% |
| 280 nm: | 16.6% | 15.2% | 21.9% | 19.5% | 11.7% | 10.8% |
| ***Tm*9D8*** | | | | | | |
| 254 nm: | 69.2% | 66.5% | 70.1% | 71.1% | 35.1% | 35.9% |
| 280 nm: | 48.1% | 47.6% | 49.9% | 48.7% | 19.3% | 20.5% |

| | Temperature (°C) | | | | | |
|---|---|---|---|---|---|---|
| | 37 | 37 | 50 | 50 | 75 | 75 |
| ***Tm*2F3** | | | **Corrected** | | | |
| 254 nm: | 8.11% | 8.22% | 23.2% | 23.5% | 20.6% | 25.3% |
| 280 nm: | 10.8% | 10.2% | 30.7% | 29.0% | 22.8% | 28.5% |
| ***Tm*9D8** | | | **Corrected** | | | |
| 254 nm: | 39.2% | 37.2% | 47.5% | 45.5% | 24.4% | 22.5% |
| 280 nm: | 42.1% | 39.6% | 50.5% | 46.9% | 32.7% | 30.7% |
| ***Tm*9D8*** | | | **Corrected** | | | |
| 254 nm: | 77.1% | 74.8% | 77.9% | 78.7% | 44.4% | 45.1% |
| 280 nm: | 76.7% | 76.4% | 77.9% | 77.1% | 46.7% | 48.6% |

**Table S2. HPLC data for reactions after 1 hour[a]**

| | HPLC yield | | | Corrected | | | | |
|---|---|---|---|---|---|---|---|---|
| **Catalyst** | **254 nm** | **254 nm** | **254 nm** | **#1** | **#2** | **#3** | **Average** | **Std. Dev.** |
| *Tm*2F3[b] | 0.834% | 0.800% | 0.840% | 1.22% | 1.17% | 1.22% | 1.20% | 0.03% |
| *Tm*9D8[b] | 3.7% | 3.7% | 4.3% | 5.4% | 5.3% | 6.1% | 5.6% | 0.4% |
| *Tm*9D8*[c] | 3.9% | 3.8% | 4.3% | 5.6% | 5.5% | 6.1% | 5.7% | 0.3% |

[a]Maximum 1000 turnovers. [b]Reactions conducted at 50 °C. [c]Reactions conducted at 37 °C.

**Table S3. HPLC data for Chart 1 and indole**

**4-bromotryptophan:**

| | HPLC yield at indicated wavelength | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Catalyst** | **254 nm** | **254 nm** | **Average** | **Corrected** | **280 nm** | **280 nm** | **Average** | **Corrected** |
| *Tm*2F3 | 58.3% | 58.1% | 58.2% | 66.5% | 60.9% | 59.7% | 60.3% | 64.6% |
| *Tm*9D8 | 63.2% | 65.7% | 64.4% | 71.9% | 67.1% | 68.0% | 67.6% | 71.1% |
| *Tm*9D8* | 69.5% | 68.6% | 69.1% | 75.8% | 72.7% | 72.2% | 72.4% | 75.3% |

**4-nitrotryptophan:**

| | HPLC yield at indicated wavelength | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Catalyst** | **254 nm** | **254 nm** | **Average** | **Corrected** | **330 nm** | **330 nm** | **Average** | **Corrected** |
| *Tm*2F3 | 3.45% | 2.51% | 2.98% | 2.52% | 1.09% | 1.14% | 1.11% | 2.58% |
| *Tm*9D8 | 1.74% | 2.70% | 2.22% | 1.87% | 1.10% | 1.08% | 1.09% | 2.53% |
| *Tm*9D8* | – | – | – | – | – | – | – | – |

**5-nitrotryptophan:**

| | HPLC yield at indicated wavelength | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Catalyst** | **254 nm** | **254 nm** | **Average** | **Corrected** | **330 nm** | **330 nm** | **Average** | **Corrected** |
| *Tm*2F3 | 16.4% | 17.9% | 17.2% | 20.9% | 19.3% | 19.4% | 19.3% | 20.9% |
| *Tm*9D8 | 4.28% | 4.97% | 4.62% | 5.68% | 4.79% | 4.92% | 4.86% | 5.3% |
| *Tm*9D8* | 5.57% | 5.80% | 5.68% | 6.98% | 6.77% | 6.97% | 6.87% | 7.5% |

**5-bromo-7-fluorotryptophan:**

| | HPLC yield at indicated wavelength | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Catalyst** | **254 nm** | **254 nm** | **Average** | **Corrected** | **280 nm** | **280 nm** | **Average** | **Corrected** |
| *Tm*2F3 | 40.8% | 42.1% | 41.4% | 34.3% | 46.8% | 48.1% | 47.4% | 34.3% |
| *Tm*9D8 | 96.8% | 97.1% | 96.9% | 94.1% | 97.1% | 97.7% | 97.4% | 94.4% |
| *Tm*9D8* | 94.4% | 95.4% | 94.9% | 91.5% | 95.1% | 96.4% | 95.7% | 91.7% |

**5-chloro-7-iodotryptophan:**

| | HPLC yield | | |
|---|---|---|---|
| **Catalyst** | **280 nm** | **280 nm** | **Average** |
| *Tm*2F3 | 2.3% | 2.2% | 2.3% |
| *Tm*9D8 | 19.7% | 20.6% | 20.2% |
| *Tm*9D8* | 18.9% | 17.8% | 18.4% |

**tryptophan:**

| | HPLC yield | | |
|---|---|---|---|
| **Catalyst** | **280 nm** | **280 nm** | **Average** |
| *Tm*2F3 | 99.3% | 97.3% | 98.3% |
| *Tm*9D8 | 90.6% | 88.3% | 89.4% |
| *Tm*9D8* | 79.0% | 79.7% | 79.3% |

**Table S4. Primers for construction of recombination libraries**

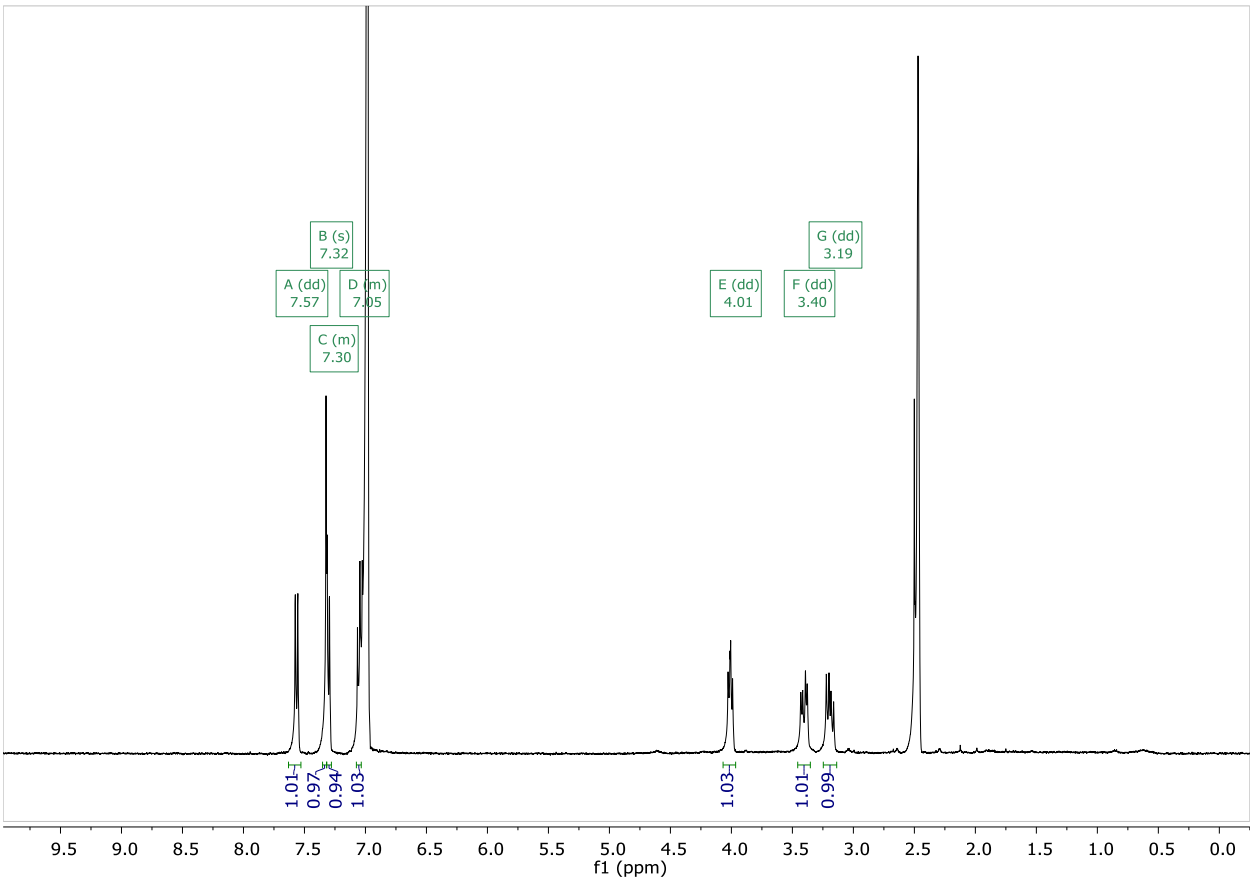
| Fragment | Forward Primer | Reverse Primer |
|---|---|---|
| Fragment 1: *Nde*I to E30 | GAAATAATTTTGTTTAACTTTAAGA AGGAGATATACATATG | CTCATCTTTCATGATTYCTTCGTA CGCAGCTTC |
| Fragment 2: E30 to I184 | GAAGCTGCGTACGAAGRAATCAT GAAAGATGAG | ACCAACCACAGAGCCGTWCACGT AATAGGTGGT |
| Fragment 3: I184 to G228 | ACCACCTATTACGTGWTCGGCTC TGTGGTTGGT | AGCGTTAGAACCACCGCYCACGC ACGCAACGAT |
| Fragment 4: G228 to *Xho*I | ATCGTTGCGTGCGTGRGCGGTGG TTCTAACGCT | GCCGGATCTCAGTGGTGGTGGTG GTGGTGCTCGAG |
| Gene Assembly | GAAATAATTTTGTTTAACTTTAAGA AGGAGATATACATATG | GCCGGATCTCAGTGGTGGTGGTG GTGGTGCTCGAG |

**Table S5. Primers for construction of site-saturation libraries**

| target *Tm*9D8 residue | forward primer | reverse primer |
|---|---|---|
| E30 | TGCGTACGAA**XXX**ATCATGAAAGATGAG | GCTTCCAGTTCTTCCAGAG |
| I184 | CTATTACGTG**XXX**GGCTCTGTGGTTGG | GTGGTCTGCAGGTTGGTA |
| G228 | TGCGTGCGTG**XXX**GGTGGTTCTA | ACGATGTAGTCCGGCAGAC |

The codon indicated as XXX is replaced with NDT, VHG, or TGG.

**4-cyanotryptophan NMR spectrum for Scheme 2**




## References

(1) Romney, D. K.; Murciano-Calles, J.; Wehrmüller, J. E.; Arnold, F. H. Unlocking Reactivity of TrpB: A General Biocatalytic Platform for Synthesis of Tryptophan Analogues. *J. Am. Chem. Soc.* **2017**, *139* (31), 10769–10776.